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Comparison of Protein-Extraction Methods for Gills of the Shore Crab, *Carcinus maenas* (L.), and Application to 2DE

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As it is well-established that protein extraction constitutes a crucial step for two-dimensional electrophoresis (2DE), this work was done as a prerequisite to further the study of alterations in the proteome in gills of the shore crab *Carcinus maenas* under contrasted environmental conditions. Because of the presence of a chitin layer, shore crab gills have an unusual structure. Consequently, they are considered as a hard tissue and represent a challenge for optimal protein extraction. In this study, we compared three published extraction procedures for subsequent applications to 2DE: the first one uses homogenization process, the second one included an additional TCA-acetone precipitation step, and finally, the third one associated grinding in liquid nitrogen (N_2) and TCA-acetone precipitation. Extracted proteins were then resolved using 1DE and 2DE. Although interesting patterns were obtained using 1DE with the three methods, only the one involving grinding in liquid N_2 and TCA-acetone precipitation led to proper resolution after 2DE, showing a good level of reproducibility at technical (85%) and biological (84%) levels. This last method is therefore proposed for analysis of gill proteomes in the shore crab.

Key Words: 2D gel electrophoresis, hard tissue, chitin, crustacean

INTRODUCTION

Numerous studies have shown that a proteome can be disturbed by a variety of physical and chemical agents.[1,2] Proteomic tools provide a global view of complex biological systems by examining protein mixtures. Thus, proteomics may reveal mechanisms involved in the responses of organisms to pollutants at the molecular level. Two-dimensional electrophoresis (2DE) techniques give insights into proteome contents, resulting from modifications of gene expression and post-translational modifications (PTMs).[1] More particularly, gel-based 2DE approaches, first described by O'Farrell,[3] are well-suited for analysis of protein PTM, partly as a result of the partial preservation of protein integrity, which is admittedly denaturated but not digested as in bottom-up approaches. This technique makes it possible to visualize and study protein isoforms[4] and to interface with other biochemical techniques, especially those based on antibodies.[5] Therefore, 2DE constitutes a useful method to explore modifications of the protein spectrum in polluted environments.

Crab species constitute pertinent models in marine environments. In particular, the shore crab, *Carcinus maenas*, is abundant and widely distributed in almost all types of shore facies in western Europe, where it occupies the intertidal and subtidal zones. Gills are the first functional organs in contact with water and therefore, with pollutants, so they represent a relevant tissue in ecotoxicological studies.[6] However, gills of decapods represent a challenge in terms of protein extraction because of the presence of a thin layer of endocuticule, mainly made of chitin, that may create interferences.[7]

In this work, we compared three methods of protein extraction reported in the literature: (1) a classical extraction protocol, (2) a second protocol that included an additional TCA-acetone precipitation step, and (3) a third protocol with a grinding in liquid nitrogen (N_2) before TCA-acetone precipitation. The efficiency of extractions was assessed by SDS-PAGE. Several criteria were considered to determine the best protein-extraction method for our model: the number of spots, their distribution, and the

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absence of drags. Finally, the reproducibility of the selected method was checked using biological and technical replicates.

MATERIALS AND METHODS

Sample Collection and Gill Dissection

Crabs were collected at low tide on the Le Havre seashore (49° 30′ N; 0° 6′ E), France. Adult males in intermolt period, with a minimum carapace size of 40 mm, were selected. Back to the laboratory, gills were rapidly collected on ice, snap-frozen in liquid N_2, and stored at −80°C until analyses could be done. A certificate from the Ethics Committee of CENOMEXA validates approval of methods used.

Protein Extraction

A summary of the three protocols outlined in this study is shown in Fig. 1.

Method A: homogenization in extraction solution

Protein extraction was adapted from Montes Nieto et al.[8] Briefly, frozen gills (300 mg) were homogenized in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 M sucrose, 5 mM magnesium acetate, 0.15 M KCl, 20 mM DTT, 2 mM EDTA, and protease inhibitor (16 μg/ml aprotinin). The homogenates were centrifuged at 10,000 *g* for 15 min at 4°C, and supernatants were collected and kept on ice for protein quantification.

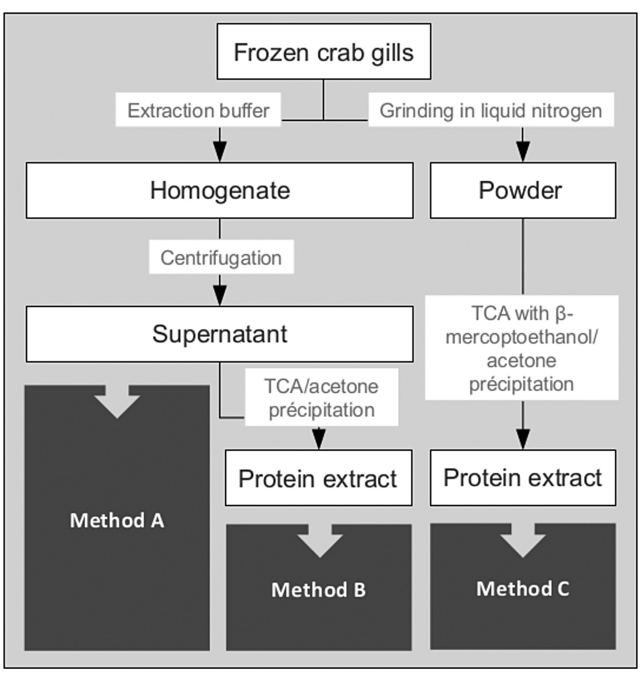

FIGURE 1

Schematic summary of the three protocols used for extraction of gill proteins from *C. maenas*.

Method B: TCA-acetone precipitation after homogenization in extraction solution

Protein extraction was carried out as described above. Then, 1 vol TCA (50%) was added to 4 vol of the collected supernatant, and the mixture was kept on ice during 45 min for protein precipitation. Subsequently, the mixture was centrifuged at 10,000 *g* for 30 min at 4°C. Supernatants were discarded, and protein pellets were washed twice with acetone and submitted to centrifugation (10,000 *g*, 15 min, 4°C). Protein pellets were finally air-dried and resolved in a solution containing 7 M urea, 20 mM Tris, pH 7.5, 2% CHAPS (w/v), and 65 mM dithioerythritol.[9]

Method C: grinding in liquid N_2, followed by TCA-acetone precipitation

Frozen gills (300 mg) were ground into a fine powder in liquid N_2 using a prechilled mortar and pestle and transferred to a 15-ml Falcon tube. Then, 5 ml of a solution, containing 10% TCA, diluted in acetone and 2% β-ME (v/v), was poured on the powder in each tube. After 2.5 h incubation at −20°C, precipitated proteins were centrifuged at 10,000 *g* for 10 min at 4°C, washed three times in acetone (10,000 *g*, 15 min, 4°C), and air-dried.[10] The pellets were resolved in a solution containing 9 M urea, 2% CHAPS (w/v), and 65 mM dithioerythritol.

Protein Measurement

Measurement of protein concentrations was done according to the Bradford method using BSA as a standard.[11]

Electrophoresis

1DE

Protein extracts (20 μg) from each extraction method were mixed with SDS loading buffer (2% SDS, 20% glycerol, 0.2 M Tris, pH 6.8) and loaded in three technical and three biological replicates onto 10% SDS-PAGE gels. Gels were run at a constant 90 V for 2 h.

2DE

For analytical gels, 300 μg proteins were mixed to reached 380 μl with a rehydratation buffer containing 9 M urea, 2% CHAPS (w/v), 65 mM dithioerythritol, and IPG buffer and loaded on 18 cm (pH 3–10) Amersham Immobiline DryStrips for overnight, passive rehydratation. For preparative gels, 1000 μg proteins were used. Strips were then subjected to IEF on Multiphor II, with an electrophoresis Power Supply EPS 3501 XL (Amersham Pharmacia Biotech, GE Healthcare, Piscataway, NJ, USA; 20°C, 83 μA/strip), by applying the following parameters: 0–500 V (linear gradient) over 10 min, 500 V for 5 h, 500–3,500 V (linear gradient) over 5 h, and 3,500 V for 9 h, 30 min. After IEF, strips were soaked in 15 mM DTT in equilibration buffer (50 mM Tris-HCl buffer, pH 6.8, containing 6 M urea, 4% SDS, 25% glycerol) for 12 min and soaked in 120 mM iodoacetamide and bromophenol blue in the equilibration buffer for 5 min. SDS-PAGE was carried out on 12% Tris-glycine gels in PROTEAN Plus Dodeca Cell apparatus (Bio-Rad, Hercules, CA, USA) at 15°C, 50 mA/gel for 15 min, 83 mA/gel for 15 min, and 20 V/gel for 6 h, or until the bromophenol blue front reached the bottom of the gels.

Gel Staining

Coomassie blue

1D gels were stained with Coomassie blue (40% ethanol, 10% acid acetic, 0.25% Brillant blue R) for 1 h and then distained in a solution containing 20% ethanol and 10% acetic acid for 1 day.

Silver nitrate

Analytical 2D gels were stained with silver nitrate. Gels were fixed overnight with 40% ethanol and 10% acetic acid and were then incubated in a solution containing 153 mM sodium acetate, 12 mM sodium thiosulfate, 30% ethanol, and 0.52% glutaraldehyde for 30 min. After three washes with water for 15 min, gels were stained in a solution containing 5 μM silver nitrate, 0.02% formaldehyde, for 40 min. Proteins were revealed by soaking gels in a development solution (240 mM sodium carbonate, 0.01% formaldehyde) for 15 min. When proteomic profiles were apparent, the development was stopped by soaking gels in a 50-mM EDTA solution for 10 min.[12]

Gel Analysis

Stained gels were scanned on a GS-800 calibrated densitometer (Bio-Rad). Then, the 2DE gel images were analyzed using Delta2D software (Decodon, Germany). Gel pictures were warped to align each spot with its corresponding spot on other gels, and intensities of the spots were scored to compare proteomic profiles.

Statistical Analysis

The evaluation of the 2DE reproducibility was carried out on three different crab samples, using three technical replicates. The coefficient of variability (CV) for spot intensity was calculated for each spot detected in all technical and biological replicates. The final proportion of spots with a CV above 0.5 was used as an indicator of reproducibility.[13]

RESULTS

Comparison of the Extraction Methods

Extraction methods were first compared by separating the samples on a 1DE gel and staining with Coomassie blue. The obtained patterns were somewhat different, according to the extraction method (Fig. 2) All three methods revealed proteins over a wide range of molecular weight (from <20 kDa to >250 kDa), even if the representation of small proteins was slightly better using a TCA precipitation step (Methods B and C). Highly abundant proteins in the 70- to 75-kDa range were observed, as expected, in every lane. Given these first results, the three methods of extraction were considered as suitable for a 2DE test.

Images of the gels obtained after 2DE and silver nitrate

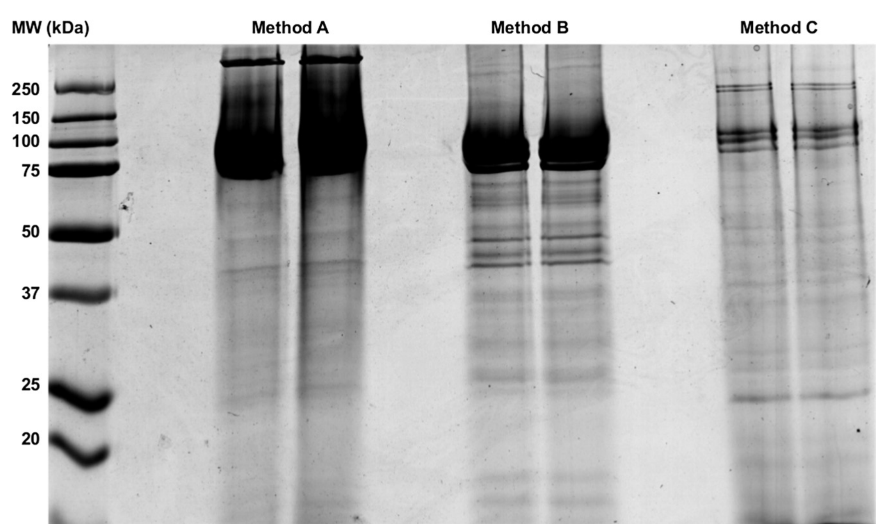

FIGURE 2

1DE comparison of extraction protocols of gill proteins in the shore crab *C. maenas*. Lanes 1 and 2 (Method A), pattern obtained after simple homogenization of gills in a extraction solution; Lanes 3 and 4 (Method B), pattern obtained after an additional step of TCA-acetone precipitation; Lanes 5 and 6 (Method C), pattern obtained by grinding tissue in liquid N_2 after TCA-acetone precipitation.

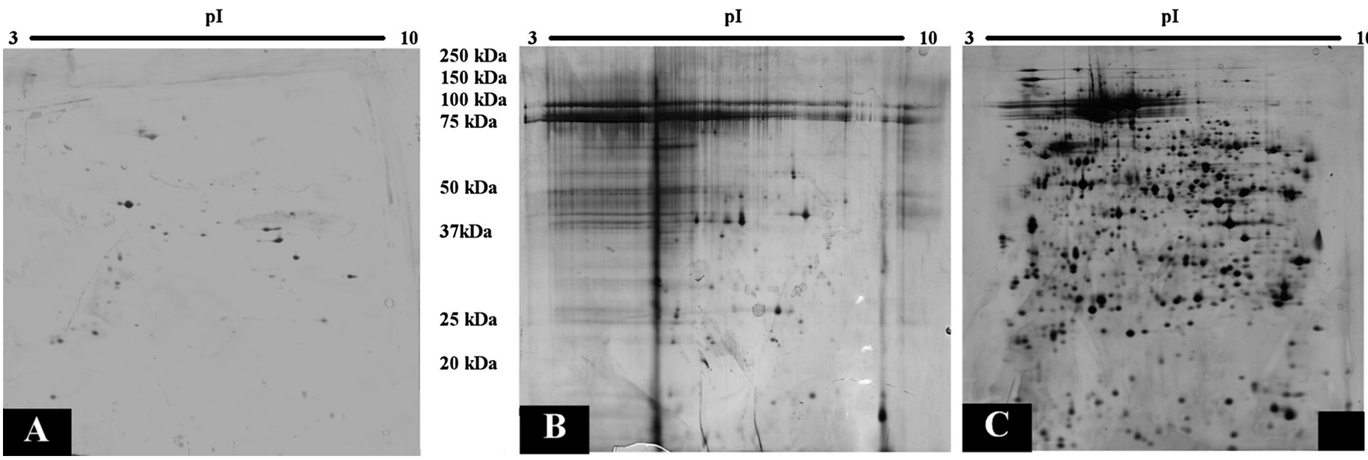

FIGURE 3

Comparison of 2DE patterns of the three extraction protocols, Panel A, B, C corresponds to Methods A, B, C respectively. For each tested method, 300 μg gill proteins of the shore crab *C. maenas* were loaded on a pH 3–10 nonlinear strip for IEF and then separated on a 12% SDS-PAGE before silver nitrate staining. See Fig. 2 legend for other indications.

staining are presented in Fig. 3. In comparison with 1DE, the spot patterns obtained with the three protein-extraction methods differed greatly. Indeed, gels corresponding to Extraction Method A showed very few spots (<200), with a main localization in the low molecular weight and acid part of the gels (Fig 3A). Method B, integrating a TCA precipitation step, revealed more spots but also agglutinations in drags: Delta2D software detected 193 well-defined spots plus a number of horizontal and vertical drags (Fig 3B). Method C led to a better and more exploitable pattern than the two others. The analysis revealed 1069 spots that were distributed all over the gel (Fig 3C), with characteristic smears at 75 kDa corresponding to the bands observed on 1DE gels at this molecular weight. Based on these observations, Method C, involving liquid N_2 and TCA-acetone precipitation, appeared to be the most suitable protocol for crab-gill protein extraction and was therefore selected for further analyses.

Reproducibility of 2DE Patterns

The reproducibility of 2DE patterns obtained with Method C was estimated by examining the technical and biological variability of spots. To this end, analyses were done on gill extracts from three different crabs (biological replicates), and each of them was performed in three replicates (technical replicates). The analysis of proteomic profiles with Delta2D (Decodon) highlighted a major conservation of protein patterns among all types of replicates (Fig. 4A). In proteomic profiles obtained from technical replicates, 89%, 85%, and 91% of spots had a CV of intensity below 0.5, respectively, for each of the three gill extracts. Figure 4B illustrates the biological reproducibility for the three crabs: 84% of spots had a CV below 0.5.

DISCUSSION

The present work points out that the unusual structure of crab gills excludes the use of classical methods of protein extraction and reveals that the best results for 2DE profiling can be obtained by coupling grinding in liquid N_2 and TCA-acetone precipitation.

The low numbers of spots observed in 2DE gels per-

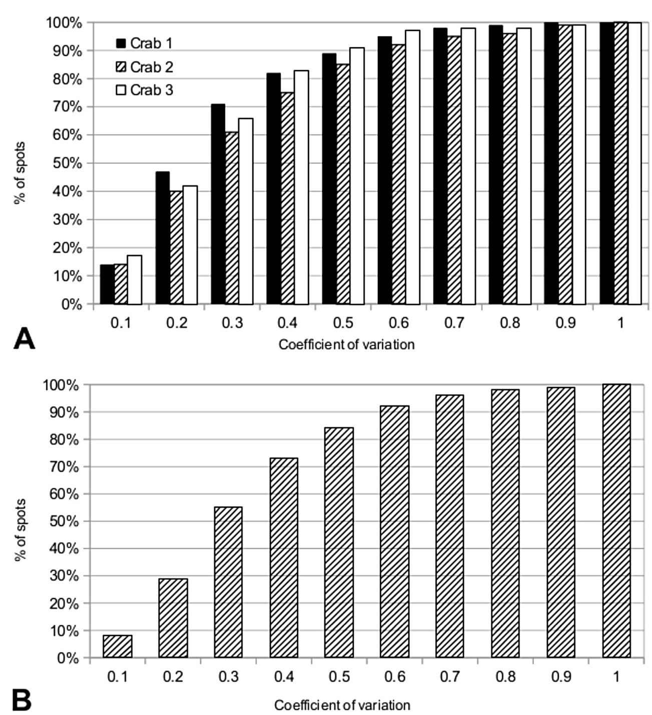

FIGURE 4

Quantitative and qualitative reproducibility of spot detection: bars represent the percentage of total spots detected according to CV (A) for the technical replicates and (B) for the biological replicates.

formed with Methods A and B compared with the relatively rich profiles obtained on 1DE in the same conditions are noticeable and undoubtedly reflect a loss of proteins during IEF. For instance, the presence of impurities could generate aggregates of protein that limit their migration from strip to 2DE gel.[14] Given the unusual composition of crustacean gill tissue, it is likely that this failure is linked to the presence of cuticle residues. Indeed, crab gills are phyllobranchiate gills, which are a stacking of several lamae consisting of a single-layer of epithelium recovered by an endocuticle.[15] Moreover, numerous horizontal drags were observed in 2DE gels performed with Method B, highlighting the presence of interfering substances in the sample. Such interferences have already been observed in carbohydrate-rich samples, where they also led to horizontal streaking.[16] The TCA-acetone precipitation included in this method apparently did not eliminate impurities from the sample. Nucleic acids are also a possible source of such interferences and can be removed by adding nuclease, such as benzonase, during the homogenization step. However, this treatment failed to provide any noticeable improvement on 2DE gels (data not shown). These streaks are probably a result of the presence of chitin residues, which are often wrongly considered as a protein, possibly as a result of a confusing name, whereas they are actually composed of N-acetylglucosamine-polymerized molecules encompassing proteins and lipids that are cross-linked to form the rigid cuticle. Chitin can be compared with plant cellulose and other structural nonproteic substances present in hard tissues, which represent challenges for extraction processes in proteomic approaches.[17] Ultracentrifugation of supernatants from Extraction Method A was first tested to eliminate small cuticle residues as a result of mechanical homogenization but did not exhibit any improvment (data not shown). In contrast with Method C, the first step of protein precipitation in a water-free condition helped to remove cellular debris by centrifugation in peculiar chitin residues to prevent electrophoresis interactions.

The divergence of results between our work and that of Montes Nieto et al.,[8] whose method inspired the first tested protocol (Method A/homogeneization), is somewhat surprising, as the method was applied on the same animal model and the same tissue. The discrepancy may come from the use of different electrophoresis and staining procedures. These authors revealed proteins with a 4–7 pH gradient, whereas in the present study, the range of pI was wider. Moreover, protein staining methods differed, and SYPRO Ruby can lead to better protein detection than the classical silver labeling.[18] Nevertheless, the sensitivity of these two staining procedures are rather similar, so it cannot explain such differences in the number of detected proteins.[4] Another major difference is that the rehydration and IEF steps were conducted separately in our assay, whereas they were performed in parallel in the previous study. However, even if such an approach could lead to the detection of ~400 spots, it remains clearly less than the 1000 spots resolved with the liquid N_2-TCA method. This would indicate that this approach is a more suitable extraction procedure to study the proteome of the crab gills.

It must be stressed that although Method C succeeded in providing an exploitable proteomic map, protein precipitation could cause a loss of proteins. Nevertheless and in agreement with literature, the TCA-acetone precipitation step gave the best patterns for most of hard tissues in 2DE and also deleted interfering substances, such as polysaccharides and/or nucleic acids, leading to a better reproducibility.[10,19] Reproducibility is an important factor for 2DE analysis but is a challenge, as a number of different parameters are involved, such as migration artifact,[4] gel polymerization, or biological variation.[20] To attest reproducibility of 2DE, Choe and Lee[13] used the CV for spot intensity and chose a threshold of 0.5 that corresponded to a quantity of >80% of the total detected spots. In our study, technical and biological replicates systematically exhibited high reproducibility, and data analyses were reinforced by the use of stringent statistical tests.[21]

As a conclusion, our work illustrates the crucial importance of the protein-extraction procedure in 2DE studies and proteomics strategy. Among the three extraction methods tested, only one, combining grinding in liquid N_2 and precipitation of proteins in TCA-acetone, delivered good patterns of crab-gill proteome after 2DE and gave a correct level of reproducibility. This extraction method will therefore be used in further works devoted to study proteome modifications in crab gills in various environmental conditions.

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DISCLOSURE

The authors declare that there is no conflict of interest.

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